US Environmental Protection Agency
Office of Pesticide Programs

Office of Pesticide Programs
Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD

Standard Operating Procedure for
OECD Quantitative Method for Evaluating Bactericidal Activity of Microbicides Used on Hard, Non-Porous Surfaces

SOP Number: MB-25-01

Date Revised: 11-29-12

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Title	OECD Quantitative Method for Evaluating Bactericidal Activity of Microbicides Used on Hard, Non-Porous Surfaces
Scope	To provide a quantitative procedure for testing the bactericidal activity of liquid antimicrobial substances against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus hirae* designed for use on hard, non-porous surfaces. This SOP is based on OECD test guidelines dated August 5, 2011 (see ref. 15.1). Details for performing the method with *Mycobacterium terrae* are provided in Attachment 2.
Application	This method measures log reduction (LR) as the quantitative measure of efficacy for liquid disinfectants on a hard nonporous surface.

	Approval Date
SOP Developer:	_____ Print Name: _____
SOP Reviewer	_____ Print Name: _____
Quality Assurance Unit	_____ Print Name: _____
Branch Chief	_____ Print Name: _____

Data SOP issued:	
Controlled copy number:	
Date SOP withdrawn:	

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1. Definitions	Additional abbreviations/definitions are provided in the text. 1. Eluate = recovered eluent that contains the test organism 2. Eluent = any liquid that is harmless to the test organism(s) and that is added to a vial containing the carrier to recover the test organism. 3. Stock culture = frozen culture used to prepare the test culture 4. Final test suspension = the test suspension with the addition of the soil load
2. Health and Safety	Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Material Safety Data Sheet for specific hazards associated with the test substance.
3. Personnel Qualifications and Training	1. A reference standard (e.g., predetermined concentrations of sodium hypochlorite) may be used to check method performance and analyst proficiency. 2. Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	Refer to SOP EQ-01, EQ-02, EQ-03, EQ-04 and EQ-05 for details on method and frequency of calibration.
5. Sample Handling and Storage	Refer to SOP MB-22, Disinfectant Sample Preparation, and SOP COC-01, Chain of Custody Procedures.
6. Quality Control	For quality control purposes, the required information is documented on the appropriate form(s) (see section 14).
7. Interferences	1. Prior to efficacy testing, verify neutralizer effectiveness using the procedure outlined in SOP MB-26 (Neutralization of Microbicidal Activity using the OECD Quantitative Method). 2. During testing, do not process carriers where the test substance runs off of the carrier; replace with new carrier(s) and vial(s).
8. Non-conforming Data	1. The mean $\log_{10}$ density for control carriers (referred to as the Test Log_{10} Density (*TestLD*)) falls outside the specified range. The *TestLD* for control carriers must be between 0.5 and 1.5 logs higher than the performance standard. a. The *TestLD* must be at least 4.5 (corresponding to a geometric mean density of 3.2×10^4) and not above 5.5 (corresponding to a geometric mean density of 3.2×10^5); a *TestLD* below 4.5 or above 5.5 invalidates the test, except for two retesting scenarios (outlined in the study protocol).

9. Data Management	Data will be archived consistent with SOP ADM-03, Records and Archives.
10. Cautions	Avoid extended soaking of the carriers in water or detergent and prolonged rinsing to reduce risk of corrosion or rusting.
11. Special Apparatus and Materials	1. Test microbes: *Pseudomonas aeruginosa* (ATCC #15442), *Staphylococcus aureus* (ATCC #6538), and *Enterococcus hirae* (ATCC #10541) 2. Culture media. Refer to SOP MB-10, Media and Reagents Used in Microbiological Assays, for QC of media and reagents. a. *Cryoprotectant solution (TSB with 15% glycerol).* Suspend 7.5 g tryptic soy broth in 212.5 mL de-ionized water. Add 37.5 g glycerol and stir, boil to homogenize. Dispense into bottles and autoclave for 15 minutes at 121°C. b. *Tryptic soy agar (TSA).* Prepare according to manufacturer's instructions. c. *TSB.* Prepare according to manufacturer's instructions. d. *Synthetic broth (SB).* Prepare according to manufacturer's instructions. 3. Reagents e. *Neutralizer in eluent.* The neutralizer is sterilized with or aseptically added to PBS with Tween-80 prior to use. The final concentration of Tween-80 in the eluent is typically 0.1% v/v. When the neutralizer is heat-sensitive, prepare sterile double-strength solutions of both the neutralizer and the Tween-80 in PBS and mix them in equal volumes. Non-PBS based neutralizers may be used as deemed necessary. f. *Phosphate buffer (PB) stock solution*. Dissolve 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) in 500 mL de-ionized water. Adjust pH to 7.2 ± 0.2 with 0.1 N NaOH or 0.1 N HCl and bring to 1000 mL with de-ionized water. Alternative phosphate buffers with the same pH may be used (e.g., commercially prepared 10X PBS solution). g. *Phosphate buffered saline (PBS).* Add 1.25 mL of phosphate buffer stock solution and 8.75 g of NaCl to a volumetric flask; fill with de-ionized water to the 1000 mL mark and mix. A pH of approximately 7.0 ± 0.5 is desirable. Sterilize by filtration or autoclaving. Alternative PBS formulations with the same pH may be used (e.g., dilute commercially prepared 10X PBS solution to 1X using de-

ionized water).

h. *Soil load.* The recommended default standard soil load to be incorporated in the test suspension is a mixture of the following stock solutions in PBS:

i. BSA: Add 0.5 g bovine serum albumin (BSA) to 10 mL of PBS, mix and pass through a 0.2 µm pore diameter membrane filter, aliquot and store at -20 ± 5°C.

ii. Yeast Extract: Add 0.5 g yeast extract to 10 mL of PBS, mix, and pass through a 0.2 µm pore diameter membrane filter, aliquot and store at -20 ± 5°C.

iii. Mucin: Add 0.04 g mucin (bovine or porcine) to 10 mL of PBS, mix thoroughly until dissolved, and autoclave (15 minutes at 121°C), aliquot and store at -20 ± 5°C.

The stock solutions of the soil load are single use only and should not be refrozen once thawed; store up to one year at -20 ± 5°C.

i. *Test substance.* Refer to SOP MB-22, Disinfectant Sample Preparation.

j. *Test substance diluent.* The test substance diluent is 375 ppm hard water (unless otherwise specified). Adjust the recipe for volumes other than 1L.

i. Prepare Solution A by dissolving 19.84 g anhydrous magnesium chloride (or 42.36 g $MgCl_2 \cdot 6H_2O$) and 46.24 g anhydrous calcium chloride ($CaCl_2$) in de-ionized water and dilute to 1000 mL. Sterilize by membrane filtration. Store the solution in the refrigerator for no longer than one month.

ii. Prepare Solution B by dissolving 35.02 g sodium bicarbonate ($NaHCO_3$) in water and dilute to 1000 mL. Sterilize by membrane filtration. Store the solution in the refrigerator for no longer than one week.

iii. To prepare 1L of 375 ppm hard water, place 600-700 mL of de-ionized water in a 1000 mL volumetric flask and add 6.0 mL of Solution A and then 8.0 mL of Solution B. Mix and add water to the flask to reach 1000 mL. The pH of the hard water should be 7.0 ± 0.2 at room temperature. If necessary, adjust the pH by using 1 N NaOH or 1 N HCl. Ensure sterility of hard water prior to use in efficacy testing.

iv. Prepare the hard water under aseptic conditions and use

within 24 h of preparation. Measure the hardness of the water using a water hardness test kit or other suitable titration method on the day of the test.

NOTE: The target hardness expressed as mg/L calcium carbonate ($CaCO_3$) is 375 mg/L +5%/-10% (338-394 ppm). Other levels of water hardness may be used as appropriate.

k. *Water*. Either de-ionized distilled water or water with equivalent quality for making reagent solutions and culture media.

l. *Tween-80 (polysorbate 80)*

4. Apparatus

a. Calibrated positive displacement pipettes (e.g., 10 µL) for carrier inoculation.

b. Micropipettes (e.g., 200 µL) for deposition of test substance on carrier.

c. Carriers: Disks (1 cm in diameter) made from 0.7 mm thick sheets of brushed and magnetized stainless steel (AISI #430). The top of the disk is brushed and has rounded edges; only the top is visually screened and inoculated. Carriers are single-use only. See Attachment 3 for complete specifications.

d. Bottle-top dispensers, squirt bottles, pre-measured volumes in tubes, or pipettes to assist in the rinsing of vials and filters.

e. Forceps, straight or curved, non-magnetic, disposable with smooth flat tips to handle membrane filters, appropriate to pick up the carriers for placement in vials.

f. Magnet strong enough to hold the carrier in place in the vial while the liquid is being poured out of it for membrane filtration.

g. Membranes (polyethersulfone) for filter sterilization and recovery, 47 mm diameter and 0.2 µm pore size. Filtration units (reusable or disposable) may be used.

h. Spectrophotometer; calibrated.

i. Sterile vials (plastic or comparable) to hold test carriers: flat bottom and wide-mouth to accommodate addition and removal of the carriers, for holding inoculated carriers to be exposed to the test substance and for accommodating neutralizer/eluent. Suitable vials should be at least 25 mm in neck diameter and hold at least 20 mL of liquid. Transparent vials are more desirable to facilitate application of 50 µL test substance or PBS and to allow for the viewing of the

	carriers for removal of inoculum. j. Certified timer k. Desiccator with desiccant (e.g., $CaCO_3$) m. Vacuum source: in-house line or suitable vacuum pump 5. Hach kit. Total hardness, 10 to 4,000 mg/L as $CaCO_3$ (Hach Digital Titrator Method 8213)
12. Procedure and Analysis	
12.1 Preparation and sterilization of carriers	a. Visually check the brushed top surface of the carriers (with the rounded edge) for abnormalities (e.g., rust, chipping, deep striations) and discard if observed. Record physical screening of carriers on form provided in section 14. b. Soak visually screened carriers in a suitable detergent solution (e.g., Liquinox) free from any antimicrobial activity for 2-4 hours to degrease and then rinse thoroughly in distilled water. c. Prior to sterilization, place up to 20 clean dry carriers on a piece of filter paper inside the bottom surface of a glass Petri dish (150 mm in diameter). Cover the Petri dish with its lid and sterilize. After sterilization, carriers may be transferred to sterile Petri dishes without filter paper for inoculation.
12.2 Preparation of test organisms	a. Refer to Attachment 1 for preparation of the frozen stock cultures for *Pseudomonas aeruginosa* (ATCC #15442), *Staphylococcus aureus* (ATCC #6538), and *Enterococcus hirae* (ATCC #10541). b. Defrost a cryovial; defrost rapidly to avoid loss in the viability of the preserved cells (e.g., expose to running water to thaw). Each cryovial is for single use only. c. *Pseudomonas aeruginosa*: Add 100 µL of defrosted stock culture to 10 mL of synthetic broth, briefly vortex mix and incubate for 18-24 h at 36 ± 1°C. d. *Staphylococcus aureus* or *Enterococcus hirae*: Add 100 µL of defrosted stock culture to 10 mL TSB, briefly vortex mix and incubate for 18-24 h at 36 ± 1°C. e. In addition, inoculate an agar plate (TSA with 5% sheep blood, BAP) with a loopful of the test culture and streak for isolation. Incubate plate with the test culture and examine for purity. Record results of purity check on microbe tracking sheet (see section 14).

	f. Following incubation, use the broth cultures to prepare a test suspension for each organism. g. For *Pseudomonas aeruginosa*, inspect culture prior to harvest; discard if pellicle has been disrupted (fragments in culture). Remove visible pellicle on surface of medium and around associated interior edges of the tube by pipetting or with vacuum suction. Using a serological pipette, withdraw the remaining broth culture (approx. 7-8 mL) avoiding any sediment on the bottom of the tube. h. Centrifuge the 18-24 h broth cultures as described below to achieve the desired level of viable cells on the dried carrier. i. Centrifuge at ~5,000 g_N for 20 ± 5 minutes and re-suspend the pellet in 10 mL PBS. Dilute or concentrate the culture appropriately to achieve the target carrier counts. Note: Remove the supernatant without disrupting the pellet. For *S. aureus*, disrupt the pellet using vortexing or repetitive tapping/striking against a hard surface to disaggregate the pellet completely prior to re-suspending it in 10 mL. If necessary, add 1 mL of PBS to the pellet to aid in the disaggregation. j. To achieve carrier counts in the range of 4.5 to 5.5 logs for *S. aureus*, resuspend the pellet in 10 mL PBS. If necessary, further dilute the culture (e.g., 1:25, 40 µL *S. aureus* + 960 µL PBS) prior to preparing the inoculum with the soil load. k. To achieve carrier counts in the range of 4.5 to 5.5 logs for *P. aeruginosa*, resuspend the pellet in 10 mL PBS. Use this culture to prepare the inoculum with the soil load. l. Optical density/absorbance (at 650nm) may be used as a tool to monitor/adjust the re-suspended test suspension.
12.3 Preparation of the final test suspension with soil load	a. Vortex the test suspension for 10-30 seconds or until re-suspended (no more than 60 seconds) to evenly distribute the cells. b. To obtain 500 µL of the final test suspension vortex each component and combine the following: i. 25 µL BSA stock ii. 35 µL yeast extract stock iii. 100 µL mucin stock iv. 340 µL test suspension
12.4 Inoculation	a. Following the addition of the soil load, vortex the final test

	and drying of carriers	suspension for 10 seconds. b. Inoculate the number of carriers required for the test plus extras. Withdraw 10 µL of the final test suspension with a calibrated positive-displacement pipette and deposit it at the center of a clean sterile screened carrier (a maximum of 20 carriers per Petri dish); avoid contact with carrier and do not spread the test suspension with the pipette tip. For consistency, use the same pipette tip to inoculate each batch of carriers. Discard any inoculated carrier where the final test suspension has run over the edge. c. Inside a biological safety cabinet, transfer the Petri dish with the inoculated carriers into a desiccator and remove the lid of the Petri dish. Close the desiccator and check that it is properly sealed. Evacuate the desiccator using a vacuum source to achieve 20-25 inches mercury (508-635 torr; 677-847 mbar; 68000-85000 Pascal). d. Hold the inoculated carriers in the evacuated desiccator at 20-25°C for 60 ± 10 minutes. If carriers are not dry within the specified time, check the desiccator system (e.g., refresh desiccant if necessary). Do not use carriers that are visibly wet.
12.5	Exposure of the dried inoculum to the test substance or PBS (control counts)	a. Evaluate four control carriers and three treated carriers for each test substance tested (one test organism and contact time/temperature combination) unless specified otherwise. One set of control carriers may be used for evaluating multiple test substances against one organism on one test day. b. Use a certified timer to ensure that each carrier receives the required exposure time (e.g., 5 min ± 3 sec). c. Using sterile forceps transfer each dried carrier with the inoculated side up to a flat-bottom vial and cap the vial. Repeat until all carriers are transferred. Note: Prior to testing, inoculated carriers can be stored at 20-25°C for up to approximately one hour after drying. d. In a timed fashion, deposit 50 µL of the test substance, equilibrated to 20-25°C, over the dried inoculum on each test carrier, ensuring complete coverage, at predetermined staggered intervals. Use a new tip for each carrier; do not touch the pipette tip to the carrier surface. Do not cap the vials. e. Hold the test carriers at 20-25°C for the selected contact period. f. Treat control carriers last – each control carrier receives 50 µL phosphate buffered saline (PBS), equilibrated to 20-25°C, instead of

	the test substance. Hold the control carriers at 20-25°C for the contact period.
12.6 Neutralization of test substance and elution of test organisms	The neutralizer for the control carriers is the same as that for the treated carriers. a. Within ± 3 seconds of the end of the contact period, add 10 mL of neutralizer at room temperature to each vial in the specified order, including controls, according to the predetermined schedule (the neutralized vial is documented as the 10^0 dilution). Briefly (2-3 sec) vortex each vial following the addition of the neutralizer. b. Following the neutralization of the entire set of carriers, vortex each vial for 30 ± 5 seconds at high speed to recover the inoculum; ensure that the carrier is vortexing along with the liquid in the vial. If possible, visually examine each carrier and, in case of incomplete elution, perform further vortexing to remove inoculum. Do not remove the carrier from the vial.
12.7 Dilution and recovery	a. Initiate dilutions within 30 min at room temperature after neutralization. Initiate filtration within 30 min of preparing the dilutions. b. If necessary, serially dilute the eluate from the 10^0 dilution (vial with the carrier) prior to filtration. c. Filter all samples, control and treated, through 0.2 µm PES membrane filters. Direct plating is not allowed. d. Pre-wet each membrane filter with approximately 10 mL of sterile PBS. Note: Use separate membrane filters for each eluate; however, the same filtration unit may be used for processing eluates from a given carrier starting with the most dilute sample first. e. For the eluate in the 10^0 dilution (10 mL, or 9 mL if dilutions were made), vortex the vial for 5 sec and holding a magnet at the bottom of the vial to keep the carrier in place, pour the eluate into the filter unit. f. Rinse the vial with ~20 mL of PBS, vortex for 5 sec and keeping magnet in place, pour the wash into the same filter unit. Repeat this step one more time. For dilution tubes, rinse each tube once with ~10 mL of PBS and briefly vortex. g. Swirl the contents of the filter unit and apply the vacuum. Note: If desired, the vacuum may be left on for the duration of the

filtration process beginning with 12.7d.

h. With the vacuum on, rinse the inside surface of the funnel unit with an additional ~40 mL PBS.

i. Aseptically remove the membrane filter and place on the recovery medium (TSA). Avoid trapping any air bubbles between the filter and the agar surface.

j. Incubate the plates at 36 ± 1°C for 24-48 h.

k. The elution steps for control carriers are the same as for the test carriers; however, eluates from control carriers will always require 10-fold dilutions to provide countable filters (up to 200 CFU/filter).

12.8 Recording results

a. Record results as CFU per carrier after 24-48 hrs of incubation. Colony counts in excess of 200 should be recorded as Too Numerous to Count (TNTC). If no colonies are present, record as zero.

b. Inspect the growth on the filters for purity and typical characteristics of the test microbe (see Table 1). Gram stain one representative colony per carrier set with growth for treated and controls. Record results on the Test Microbe Confirmation Sheet. Isolation streaks may be performed for additional verification of the test organism.

Table 1. General diagnostic characteristics for *P. aeruginosa*, *S. aureus*, and *E. hirae* (see ref. 15.7, 15.8, 15.9, and 15.10)

Aspect	*P. aeruginosa**	*S. aureus**	*E. hirae**
Gram stain reaction	Negative	Positive	Positive
Mannitol Salt Agar	No Growth	Circular, small, yellow colonies, agar turning fluorescent yellow	No Growth
Cetrimide Agar	Circular, small, initially opaque, turning fluorescent green over time; agar fluorescent yellowish green	No Growth	No Growth
Blood agar (BAP)	Flat, opaque to off-white, round spreading (1), metallic sheen, slightly beta hemolytic	Small, circular, yellow or white, glistening, beta hemolytic	Round gray colonies, slightly alpha hemolytic
Typical Microscopic Characteristics			

	<table><tr><td>Cell appearance</td><td>Straight or slightly curved rods, single polar flagella, rods formed in chains</td><td>Spherical, occurring singly, in pairs and tetrads, sometimes forming irregular clusters</td><td>Ovoid, occurring singly, in pairs or in short chains</td></tr></table> *After 24±2 hours (1) Test organism may display three colony types: a) circular, undulate edge, convex, rough and opaque; b) circular, entire edge, convex, smooth and translucent; c) irregular, undulate edge, convex, rough, spreading, and translucent. Pyocyanin is not produced.
13. Data Analysis/ Calculations	1. All colony counts are recorded and used in calculations to determine log reductions. 2. To calculate the CFU/carrier use the following equation: $\left(\frac{CFU\ \ for\ 10^{-y} + CFU\ \ for\ 10^{-z}}{(a\times 10^{-y}) + (b\times 10^{-z})}\right)\times c$, where 10^{-y} and 10^{-z} are the dilutions filtered, "a" and "b" are the volumes filtered at each dilution (typically 9 or 10 mL), and "c" is the volume of medium originally in the vial with the carrier (10 mL). 3. Calculate the log density of each carrier by taking the $\log_{10}$ of the density (per carrier). 4. Calculate the mean $\log_{10}$ density across treated carriers. 5. Calculate the mean $\log_{10}$ density across control carriers. 6. Calculate the $\log_{10}$ reduction (LR) for treated carriers: $\log_{10}$ reduction = mean $\log_{10}$ control – mean $\log_{10}$ treated
14. Forms and Data Sheets	1. Attachment 1: Procedures for Maintenance of Bacterial Cultures – Preparation of Frozen Stock Cultures 2. Attachment 2: Procedures for Maintenance of *Mycobacterium terrae* Culture – Preparation of Frozen Stock Culture and Test Culture 3. Attachment 3: Carrier Specifications 4. Attachment 4: Confirmation Flow Charts for *S. aureus*, *P. aeruginosa*, and *E. hirae* 5. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Physical Screening of Carriers Record Form — MB-25-01_F1.docx OECD Method for Bactericidal Activity: Organism Culture Tracking Form — MB-25-01_F2.docx OECD Method for Bactericidal Activity: Test — MB-25-01_F3.docx

	Microbe Confirmation Sheet (Quality Control) OECD Method for Bactericidal Activity: Test Information Sheet MB-25-01_F4.docx OECD Method for Bactericidal Activity: Time Recording Sheet MB-25-01_F5.docx OECD Method for Bactericidal Activity: Serial Dilution Plating/Tracking Form MB-25-01_F6.docx OECD Method for Bactericidal Activity: Results Sheet MB-25-01_F7.docx OECD Method for Bactericidal Activity: Test Microbe Confirmation Sheet MB-25-01_F8.docx
15. References	1. Draft Test Guideline: Quantitative Method for Evaluating Bactericidal Activity of Microbicides Used on Hard Non-Porous Surfaces (August 5, 2011) 2. ASTM Standard E2197-11, 2011, "Standard Quantitative Disk Carrier Test Method for Determining the Bactericidal, Virucidal, Fungicidal, Mycobactericidal and Sporicidal Activities of Liquid Chemical Germicides," ASTM International, West Conshohocken, PA. 3. Package Insert – Gram Stain Kit and Reagents. Becton, Dickinson and Company. Part no. 882020191JAA. Revision 07/2011. 4. Package Insert – Catalase Reagent Droppers. Becton, Dickinson and Company. Part no. L001237. Revision 06/2010. 5. Package Insert – Staphaurex Plus*. Remel. Part no. R30950102. Revised 11/23/07. 6. Package Insert – Oxidase Reagent Droppers. Becton, Dickinson and Company. Part no. L001133. Revision 06/2010. 7. Krieg, Noel R. and Holt, John G. 1984. Bergey's Manual of Systematic Bacteriology Volume 1. Williams & Wilkins, Baltimore, MD. *P. aeruginosa* p. 164. 8. Sneath, P., Mair, N., Sharpe, M.E., and Holt, J. eds. 1986. Bergey's Manual of Systematic Bacteriology Volume 2. Williams & Wilkins, Baltimore, MD. *S. aureus* p. 1015. 9. Winn, Jr., Washington, et al eds. 2006. Koneman's Color Atlas and Textbook of Diagnostic Microbiology Sixth Edition. Lippincott Williams & Wilkins, Baltimore, MD. *E. hirae* p. 714. 10. De Vos, Paul, et al eds. 2009. Bergey's Manual of Systematic

	Bacteriology Volume 3. Springer, New York, NY. *E. hirae* p. 594.

Attachment 1

Procedures for Maintenance of Vegetative Bacterial Cultures – Preparation of Frozen Stock Cultures

A1. Preparation of Frozen Stock Cultures. Refer to SOP MB-02 for establishment of the organism control number.

a. Initiate new stock cultures from lyophilized cultures of *Pseudomonas aeruginosa* and *Staphylococcus aureus* from ATCC at least every 18 months.

b. Open ampule of freeze dried organism per manufacturer's instructions. Using a tube containing 5-6 mL of TSB, aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture. Aseptically transfer the entire rehydrated pellet back into the original tube of broth. Mix thoroughly. Incubate broth culture at 36 ± 1ºC for 24 ± 2 hours.

c. After incubation, streak a loopful of the suspension on TSA to obtain isolated colonies. Incubate the plates for 18-24 h at 36 ± 1°C. Refer to section A2 for QC of stock cultures.

d. Select 3-5 isolated colonies of the test organism and re-suspend in 1 mL of TSB. For *S. aureus*, select only golden yellow colonies. Multiple phenotypes are present for *P. aeruginosa* – the stock culture should be representative of all phenotypes present on the streak isolation plate. Spread plate 0.1 mL of the suspension on each of 6-10 TSA plates. Incubate the plates for 18-24 h at 36 ± 1°C.

e. Following the incubation of the agar plates from A1d, place approximately 5 mL sterile cryoprotectant solution on the surface of each plate. Re-suspend the growth in the cryoprotectant solution using a sterile spreader without damaging the agar surface. Aspirate the suspension from the plate with a pipette and place it in a sterile vessel large enough to hold about 30 mL. Repeat the growth harvesting procedure with the remaining plates and continue adding the suspension to the vessel (more than 1 tube may be used if necessary). Mix the contents of the vessel(s) thoroughly; if more than 1 vessel is used, pool the vessels prior to aliquoting culture.

f. Immediately after mixing, dispense 0.5-1 mL aliquots of the harvested suspension into cryovials; these represent the frozen stock cultures.

g. Store the cryovials at -70ºC or lower for a maximum 18 months then reinitiate with a new lyophilized culture.

A2. QC of Stock Cultures.

a. Conduct QC of the pooled culture concurrently with freezing. Streak a loopful on a plate of BAP. In addition, streak a loopful onto both MSA and Cetrimide. Incubate all plates at 36 ± 1ºC for 24 ± 2 hours.

b. Following the incubation period, record the colony morphology as observed on the BAPs and selective media plates (including the absence of growth) and Gram stain. See Table 1 for details on cell and colony morphology, colony characteristics on selective media, and stain reactions.

c. For each organism, perform a Gram stain (refer to 15.3) from growth taken from the BAPs according to the manufacturer's instructions. Observe the Gram reaction by using brightfield microscopy at 1000X magnification (oil immersion).

d. For additional biochemical and antigenic analyses, refer to 15.4-15.5 for *S. aureus* and 15.6 for *P. aeruginosa*.

e. Record all confirmation results on the Test Microbe Confirmation Sheet (Quality Control) (see section 14).

Attachment 2

Procedures for Maintenance of *Mycobacterium terrae* Culture – Preparation of Frozen Stock Culture and Test Culture

A1. Materials and reagents

a. *Growth medium*. Middlebrook 7H9 broth containing glycerol and 10% ADC Enrichment (MADC, 4.7 g Middlebrook 7H9 broth powder, 150 mL glycerol, 750 mL water, sterilize in autoclave. Add under aseptic conditions, 100 mL Middlebrook ADC enrichment and then add sterilized water up to 1,000 mL. The pH of the medium should be 6.6±0.2).

b. *Recovery medium*. Middlebrook 7H11 Agar.

c. *Sterile Bijou bottles.* Glass with aluminum caps; capacity 5-7 mL, with 10 glass beads (3-5 mm in diameter).

A2. Preparation of Frozen Stock Cultures. Refer to SOP MB-02 for establishment of the organism control number.

a. Initiate new stock cultures from lyophilized cultures of *Mycobacterium terrae* (ATCC 15755) from ATCC at least every 18 months. Open ampule of freeze dried organism per manufacturer's instructions. Using a tube containing 6 mL of Middlebrook 7H9 Broth with 10% ADC enrichment (MADC), aseptically withdraw 1.0 mL and rehydrate the lyophilized culture. Aseptically transfer the entire rehydrated pellet back into the original tube of broth. Mix thoroughly.

b. Spread 0.1 mL of the test organism suspension onto approximately 6-10 M7H9 or M7H11agar plates. Incubate for 20-22 days at 36±1°C. Refer to section A3 for QC of frozen stock cultures.

NOTE: Each plate will yield ~10 mL of harvested suspension and consequently nine to ten cryovials, each containing >1 mL of frozen stock culture.

c. At the end of the incubation period, add 5 mL MADC to the surface of each agar plate. Re-suspend the cells in MADC using a sterile spreader without damaging the agar surface. Aspirate the suspension from the plate with a pipette and place it in a sterile vessel large enough to hold about 30 mL. Repeat by adding another 5 mL of MADC to the agar plates, re-suspend the cells, aspirate the suspension and pool with the initial cell suspension. Repeat the growth harvesting procedure with the remaining plates and continue adding the suspension to the vessel (more than 1 vessel may be used if necessary). Mix the contents of the vessel(s) thoroughly; if more than 1 vessel is used, pool the vessels prior to aliquoting the culture.

d. Immediately after mixing, dispense >1 mL aliquots of the harvested suspension into separate cryovials; these represent the frozen stock cultures.

e. Store the cryovials at -70ºC or lower for a maximum of 18 months (from the date of harvesting/freezing).

A3.	QC of Stock Cultures.
	a. Conduct QC of the pooled culture concurrently with freezing. Streak a loopful on M7H9 or M7H11 agar and incubate at 36 ± 1ºC for 20-22 days.
	b. Following the incubation period, record the colony morphology as observed on the plates. *M. terrae* typical colony morphology includes irregular margins, and appears rough, erose, buff, opaque, and umbonate.
	c. Record all confirmation results on the Test Microbe Confirmation Sheet (Quality Control) (see section 14).
A4.	Preparation of test organisms from frozen stock cultures
	a. Defrost a cryovial; defrost rapidly to avoid loss in the viability of the preserved cells (e.g., expose to running water to thaw). Each cryovial is for single use only.
	b. Add 1 mL thawed culture to a flask of 100 mL MADC. Inoculate 1-2 flasks using a separate cryovial for each flask and incubate at 36±1°C for 20-22 days.
	c. Aliquot 25 mL portions of the 20-22 day-old MADC broth culture into each of 2-50 mL conical screw cap tubes and centrifuge at 10,000g_N for 20±5 minutes.
	d. Carefully remove the supernatant and re-suspend each pellet in 25 mL sterile distilled/de-ionized (DI) water.
	e. Centrifuge the tubes a second time at 10,000g_N for 20±5 minutes. After centrifuging, re-suspend the pellets in a total of 5 mL sterile DI water (1/10 of the starting volume), pool, and place in a bijou bottle (or equivalent) with 10 glass beads; vortex for 5 min.
	f. The approximate titer of each freshly prepared and homogenized microbial test suspension may be estimated spectrophotometrically at 650 nm, based on a standard curve specific to the test organism.
	g. For mycobacterial claims, based on a potential performance standard of a log reduction of 4, control counts should be within 4.5-5.5 logs per carrier.
	h. Prior to inoculation of carriers, aseptically add the soil load.
A5.	Refer to sections 12.3 through 12.7h for preparation of the inoculum with soil, carrier inoculation, and exposure of the carriers to the test substance or control fluid.
A6.	For recovery of *M. terrae* test culture from exposed and control carriers, use M7H11 agar plates. Incubate plates at 36±1°C for up to 21-28 days; however, monitor filters for growth and assess the number of visible colonies beginning at 12-14 days. If no colonies are visible at the end of 14 days of incubation, re-incubate the plates for an additional 7-14 days before recording the final colony counts.

Attachment 3

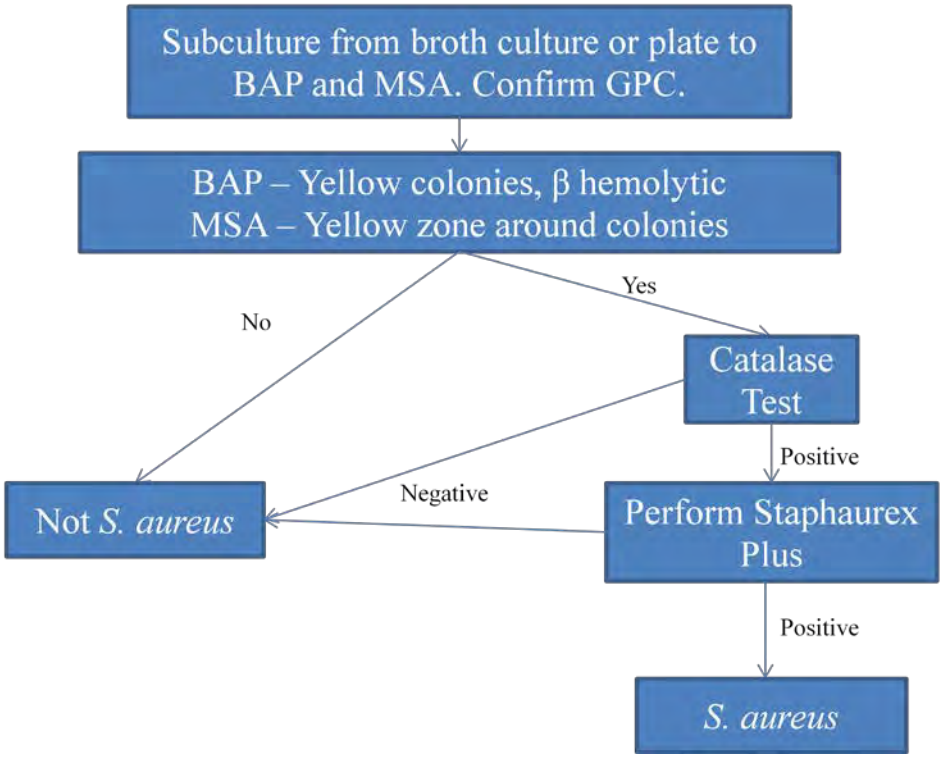
Carrier Specifications

- Ferritic stainless steel: Consist of chromium (17%) and iron and essentially nickel-free.
- AISI Type 430 (European equivalent name X6Cr17 and number 1.4016) belongs to Group 2, which is the most widely used family of ferritic alloys.
- Dimensions: 1 cm (0.39370 inch) in diameter; 0.7 mm (0.27559 inch) thick.
- AISI 430 - ASTM A240; Japanese Industrial Standard (JIS) G4305; EN 10088-2
- No. 4 Finish (EN 10088-2 1J/2J): A ground unidirectional finish obtained with 150 grit abrasive (AISI).
- Passivation: A soak in a mild acid bath for a few minutes to remove any impurities and accumulated debris from the disk surface.
- Tumbling: To remove the punching burrs from the edges of the discs they are tumbled in a barrel together with ceramic chips and a cleanser.

Attachment 4

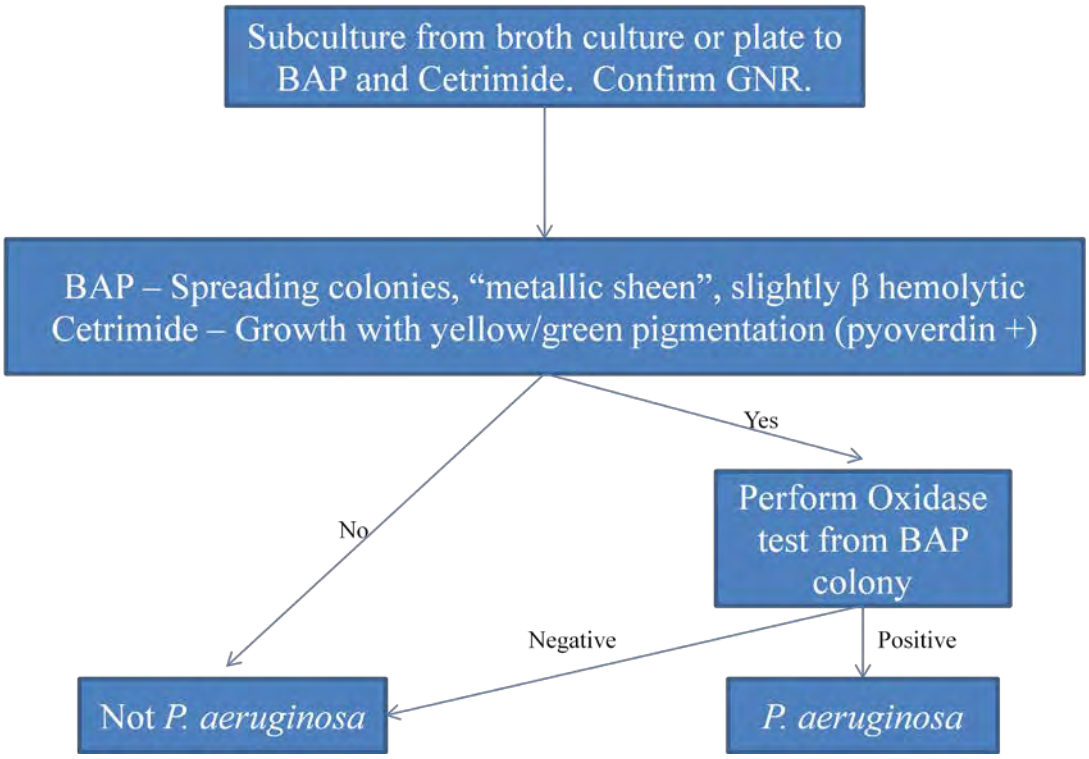
Confirmation Flow Charts for *S. aureus*, *P. aeruginosa*, and *E. hirae*

S. aureus Identification



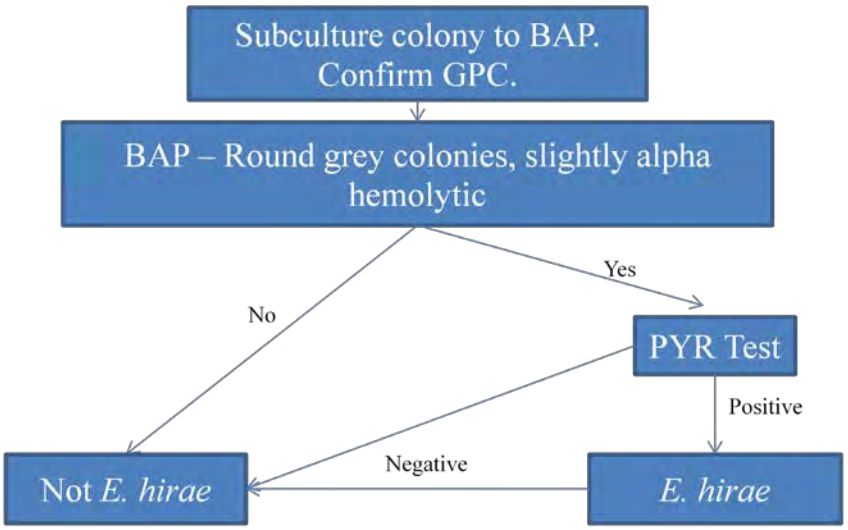
Attachment 4 (continued)

P. aeruginosa Identification



Attachment 4 (continued)

E. hirae Identification



PYR = pyrrolidonyl arylamidase